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Functional Genomic Analysis of Apoptotic DNA Degradation in *C. elegans*

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Summary

Chromosomal DNA degradation is critical for cell death execution and is a hallmark of apoptosis, yet little is known about how this process is executed. Using an RNAi-based functional genomic approach, we have identified seven additional cell death-related nucleases (*crn* genes), which along with two known nucleases (CPS-6 and NUC-1) comprise at least two independent pathways that contribute to cell killing, and likely signaling for phagocytosis, by degrading chromosomal DNA. Several *crn* genes have human homologs that are important for RNA processing, protein folding, DNA replication, and DNA damage repair, suggesting dual roles for CRN nucleases in cell survival and cell death. It should now be possible to systematically decipher the mechanisms of apoptotic DNA degradation.

Introduction

Programmed cell death (apoptosis) is important for development and tissue homeostasis of metazoans (Steller, 1995; Vaux and Korsmeyer, 1999). One critical step in apoptosis is the fragmentation of chromosomal DNA at internucleosomal regions, leading to the generation of approximately 180 bp DNA ladders (Wyllie, 1980; Zhang and Xu, 2002). Several nucleases have been implicated in mediating this chromosome fragmentation process, including DFF40/CAD (40 kDa DNA fragmentation factor/caspase-activated deoxyribonuclease) (Enari et al., 1998; Liu et al., 1997, 1998) and mitochondrial endonuclease G (Li et al., 2001; Parrish et al., 2001). DFF40/CAD normally associates tightly with its cognate inhibitor DFF45/ICAD (inhibitor of CAD) but is released from DFF45/ICAD and thus activated during apoptosis as a result of caspase cleavage of DFF45/ICAD. On the other hand, EndoG is released from mitochondria and translocates to nuclei during apoptosis where it induces DNA fragmentation through a caspase- and DFF40-independent pathway (Li et al., 2001), indicating that multiple DNA degradation pathways exist. Consistent with the observation that DFF40 and EndoG act in different pathways, mice lacking DFF40/CAD activity appear to develop normally and have normally occurring apoptosis, despite a significant reduction in DNA fragmentation in apoptotic cells (McIlroy et al., 2000; Samejima et al., 2001; Zhang et al., 1998). In addition, several other mammalian proteins, including apoptosis-inducing factor (AIF), DNase II, Topoisomerase II, and cyclophilins have been implicated in mediating apoptotic DNA degradation, mostly based on in vitro studies (Zhang and Xu, 2002). It is unclear whether these proteins are important for apoptosis in vivo. Nevertheless, these studies suggest that the apoptotic DNA degradation process is likely more complicated and tightly regulated than originally anticipated.

In the nematode *C. elegans*, at least two nucleases have been shown to mediate apoptotic DNA degradation: NUC-1, a worm type II DNase (Wu et al., 2000), and CPS-6, the *C. elegans* ortholog of EndoG (Parrish et al., 2001). Loss-of-function mutations in either *cps-6* or *nuc-1* result in accumulation of TUNEL-positive nuclei in mutant embryos, indicating that both genes function to resolve 3′OH DNA breaks (labeled by TUNEL) generated during apoptosis (Parrish et al., 2001; Wu et al., 2000). Additionally, in the *cps-6* mutant, cell deaths are delayed and sometimes blocked (in sensitized genetic backgrounds), suggesting that the DNA degradation process is important for apoptosis (Parrish et al., 2001). Unlike *cps-6*, *nuc-1* appears to be dispensable for apoptosis and likely acts in a different DNA degradation pathway since *cps-6; nuc-1* double mutants have higher numbers of TUNEL-positive cells than those of either mutant alone (Parrish et al., 2001). Recently, WAH-1, a worm AIF homolog, was found to associate with and cooperate with CPS-6 to promote DNA degradation in *C. elegans* (Wang et al., 2002), suggesting that additional factors are likely involved in regulating apoptotic DNA degradation in *C. elegans*. However, neither *nuc-1* or *cps-6* mutants nor *wah-1*(RNAi) animals display easily detectable phenotypes that would encourage additional genetic screens for mutants with similar cell death defects. Thus it becomes imperative to develop a more powerful and systematic method to identify molecular components that are involved in apoptotic DNA degradation.

RNA-mediated interference (RNAi), which can specifically abolish or reduce target gene expression (Fire et al., 1998), when used in combination with information derived from the completed genome sequence in *C. elegans* (The *C. elegans* Sequencing Consortium, 1998), has greatly facilitated functional genomic analyses, leading to the assignment of biological functions to many open reading frames (ORFs) on *C. elegans* Linkage Group I and III (Fraser et al., 2000; Gonczy et al., 2000). Additionally, candidate-based, genome-wide analyses based on a specific, assayable biological activity or biochemical property have helped identify important genes and construct protein interaction maps for complicated biological processes such as *C. elegans* vulval development and the DNA damage response (Boulton et al., 2002; Walhout et al., 2000). To systematically identify genes functioning in apoptotic DNA degradation in *C. elegans*, we conducted a candidate-based functional genomic screen using a combination of RNAi and TUNEL techniques.

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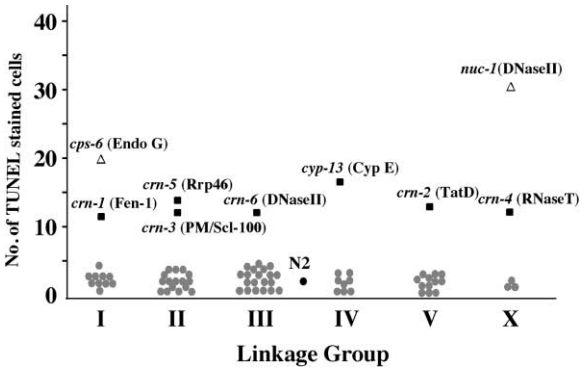

Figure 1. Seven Additional Apoptotic Nucleases Were Identified from the *C. elegans* Genome

The 77 ORFs screened for TUNEL phenotypes following RNAi treatment are categorized based on their chromosomal positions (Linkage Group; *x* axis) and plotted according to the number of TUNEL-positive nuclei (on average) detected in 1.5-fold wild-type embryos (N2) treated with RNAi (*y* axis). RNAi of 68 ORFs (gray circles) resulted in TUNEL phenotypes that were not significantly different from those of N2 animals treated with control(RNAi) (black circle). RNAi of 9 ORFs resulted in significantly higher numbers of TUNEL-positive nuclei, including two genes (*cps-6* and *nuc-1*) previously known to be involved in apoptotic DNA degradation (triangles), six new *crn* genes (cell death *r*elated *n*ucleases), and *cyp-13*, which was previously named (all in squares). The identity of the corresponding mammalian homolog is indicated in parentheses next to each of the *C. elegans* genes.

Results

Candidate-Based RNAi Screen Identified Seven Additional Cell Death Related Nucleases

We tested *C. elegans* deoxyribonucleases and ribonucleases as well as cyclophilins and topoisomerases for potential roles in apoptotic DNA degradation (Zhang and Xu, 2002). Using INTERPRO and PFAM motif searches (Apweiler et al., 2001; Sonnhammer et al., 1997), we identified 77 ORFs that fit into these categories. We conducted RNAi experiments on these 77 ORFs in three different genetic backgrounds, wild-type (N2), the *cps-6(sm116)* mutant, or the *nuc-1(e1392)* mutant, and looked for an increase or decrease of TUNEL staining in these RNAi-treated animals versus control(RNAi)-treated animals (see Supplemental Table S1 at http://www.molecule.org/cgi/content/full/11/4/987/DC1 and Experimental Procedures). RNAi of nine of these ORFs gave rise to TUNEL phenotypes that are indicative of their involvement in apoptotic DNA degradation (Figure 1, Table 1, and Supplemental Figure S1 at http://www.molecule.org/cgi/content/full/11/4/987/DC1). Two of the ORFs, C07B5.5 (*nuc-1*) and C41D11.8 (*cps-6*), were previously known to function in DNA degradation, demonstrating the effectiveness of this screen (Parrish et al., 2001; Sulston, 1976; Wu et al., 2000). The other seven ORFs were not noted before for their apoptotic phenotypes, including six previously uncharacterized genes that we have named *crn* (*c*ell death *r*elated *n*uclease) (Table 1) and one cyclophilin homolog (*cyp-13*). RNAi of each of these nine genes led to an accumulation of TUNEL-positive nuclei that can be suppressed by a strong loss-of-function *ced-3*(*n2433*) mutation (Table 1) or a strong loss-of-function *ced-4*(*n1162*) mutation (data not shown), which blocks most cell deaths in nematodes (Ellis and Horvitz, 1986), indicating that these genes are involved in apoptotic DNA degradation (Table 1). Furthermore, RNAi of *crn-2* or *crn-3* but not the other *crn* genes or *cyp-13* significantly enhanced the TUNEL phenotype of the *cps-6(sm116)* mutant, indicating that *crn-2* and *crn-3* function at least partially independently of *cps-6* (Table 1). However, double RNAi of *crn-2* and *crn-3* in N2, *cps-6(sm116),* or *nuc-1(e1392)* animals did not result in stronger TUNEL phenotype than either RNAi treatment alone, suggesting that *crn-2* and *crn-3* may function in the same pathway to mediate apoptotic DNA degradation (data not shown). Finally, RNAi of each of the seven new genes enhanced the TUNEL phenotype of *nuc-1*, suggesting that *nuc-1* functions in a different DNA degradation process from these seven genes (Table 1).

CRN Nucleases Have Mammalian Homologs

Sequence analysis of the *crn* genes and *cyp-13* revealed insightful information regarding their functions (see Supplemental Figure S1 at http://www.molecule.org/cgi/content/full/11/4/987/DC1). *crn-1* encodes a homolog of flap endonuclease 1 (FEN-1), a mammalian nuclease important for DNA replication and damage repair (Harrington and Lieber, 1994; Lieber, 1997), which we found may represent a critical switch between DNA replication/repair and apoptotic DNA degradation during apoptosis (our unpublished data). *crn-1*(RNAi) causes embryonic lethality in *C. elegans* (see Supplemental Table S1 at http://www.molecule.org/cgi/content/full/11/4/987/DC1), suggesting that it is important for cell survival and may be involved in DNA replication/repair in *C. elegans* like FEN-1. *crn-2* encodes a homolog of the TatD nuclease (see Supplemental Figure S1A at http://www.molecule.org/cgi/content/full/11/4/987/DC1), a poorly characterized *E. coli* magnesium-dependant nuclease (Wexler et al., 2000). Mammalian TatD homologs exist but have no known function (our unpublished data); our findings suggest a possible role for TatD-like nucleases in mammalian apoptosis.

crn-3 and *crn-5* encode homologs of the 100 kDa polymyositis/scleroderma autoantigen (PM/Scl-100) and Rrp46 (see Supplemental Figures S1B and S1D at http://www.molecule.org/cgi/content/full/11/4/987/DC1) (Brouwer et al., 2001), respectively, both of which are ribonuclease components of the exosome, a multi-exonuclease complex. The exosome functions in processing or degradation of several types of RNAs and is crucial for the survival of yeast cells (Perumal and Reddy, 2002). Therefore, *crn-3* and *crn-5* appear to be shared components of two different machineries for RNA processing and for apoptotic DNA fragmentation. Both *crn-3*(RNAi) and *crn-5*(RNAi) caused retarded growth of treated animals; in the case of *crn-3*(RNAi), a low penetrance of embryonic lethality was observed (see Supplemental Table S1 at http://www.molecule.org/cgi/content/full/11/4/987/DC1). These observations are consistent with their potential roles in RNA processing as components of the exosome in *C. elegans*. Interestingly, *crn-3* and *crn-5* are adjacent ORFs in a polycistronic locus, and the expression of both genes is likely regulated by the same promoter (Blumenthal et al., 2002). Thus, *crn-3* and *crn-5* might play a role in coordinating the two differ-

Table 1. TUNEL Analysis of *C. elegans* Genes Involved in Apoptotic DNA Degradation

		Strain Treated by RNAi							
		N2		*ced-3(n2433)*		*cps-6(sm116)*		*nuc-1(e1392)*	
ORF	Gene	TUNEL	n	TUNEL	n	TUNEL	n	TUNEL	n
Control		2.3 ± 0.5	16	0.3 ± 0.2	15	22.1 ± 0.5	15	35.2 ± 0.5	15
C41D11.8	*cps-6*	19.9 ± 0.5	15	1.7 ± 0.3	15	20.8 ± 0.7	15	46.7 ± 0.7	15
C07B5.5	*nuc-1*	30.9 ± 0.7	25	0.7 ± 0.3	15	43.7 ± 0.8	15	34.5 ± 0.5	15
Y47G6A.8	*crn-1*	11.3 ± 0.7	15	2.1 ± 0.6	18	21.5 ± 0.9	10	45.9 ± 0.9	15
CD4.2	*crn-2*	13.3 ± 0.8	16	1.1 ± 0.3	20	27.8 ± 0.6	16	48.9 ± 0.7	14
C14A4.4	*crn-3*	11.8 ± 0.8	16	0.8 ± 0.3	13	28.2 ± 0.8	15	47.4 ± 0.7	20
AH9.2	*crn-4*	12.2 ± 0.9	16	1.1 ± 0.3	12	18.9 ± 0.6	16	45.8 ± 0.7	15
C14A4.5	*crn-5*	13.9 ± 0.9	13	1.5 ± 0.3	13	19.7 ± 0.6	16	42.6 ± 1.6	18
K04H4.6a	*crn-6*	11.8 ± 0.7	17	1.5 ± 0.3	18	20.4 ± 0.6	15	45.2 ± 0.7	20
Y116A8C.34	*cyp-13*	16.8 ± 0.8	15	0.6 ± 0.2	16	19.1 ± 0.7	16	49.0 ± 0.9	15

The TUNEL assays were carried out as previously described (Parrish et al., 2001). TUNEL-reactive nuclei were scored in 1.5-fold stage embryos. "n" indicates the number of embryos scored. Data represent averages ± one standard error of the mean (SEM).

ent DNA degradation pathways that they represent (see below).

crn-4 is homologous to a family of 3′ to 5′ exonucleases including ribonuclease T and the epsilon subunit of DNA polymerase III (see Supplemental Figure S1C at http://www.molecule.org/cgi/content/full/11/4/987/DC1), which are involved in tRNA processing and DNA replication, respectively (Koonin and Deutscher, 1993). Like *nuc-1*, *crn-6* encodes a type II DNase (see Supplemental Figure S1E at http://www.molecule.org/cgi/content/full/11/4/987/DC1). *crn-6* and *nuc-1* may function like an acid DNase implicated in degrading DNA from apoptotic cells engulfed by macrophages (McIlroy et al., 2000). Finally, *cyp-13* is most similar to mammalian cyclophilin E (see Supplemental Figure S1F at http://www.molecule.org/cgi/content/full/11/4/987/DC1), and both have a putative RNA-recognition motif (RRM) at the amino terminus and a peptidyl-prolyl *cis-trans* isomerase domain at the carboxyl terminus (Andreeva et al., 1999; Mi et al., 1996). Cyclophilins have been implicated in apoptotic DNA degradation in mammalian cells (Montague et al., 1997), and like some other cyclophilins, we found that CYP-13 is a nuclease in vitro, suggesting that CYP-13 may directly mediate DNA degradation (see Supplemental Figure S2 at http://www.molecule.org/cgi/content/full/11/4/987/DC1). Although the function of cyclophilin E is not understood, cyclophilins have generally been implicated in facilitating cellular protein folding (Andreeva et al., 1999). Furthermore, recent studies have shown that cyclophilin E is a component of the human spliceosome (Zhou et al., 2002), and therefore, *cyp-13* may additionally be involved in RNA splicing in *C. elegans* (T. Blumenthal, personal communication). In summary, the identification of *crn-6* and *cyp-13* from our screen confirms previous observations that their mammalian counterparts are likely involved in apoptotic DNA degradation in vivo, and the identification of the other five genes (*crn-1* to *crn-5*) may reveal novel functions for their mammalian homologs in apoptosis.

CRN Nucleases Affect Progression of Apoptosis in *C. elegans*

cps-6 is important for normal progression of apoptosis, whereas *nuc-1* appears dispensable for cell death (Parrish et al., 2001). We thus examined whether *cyp-13* and the six *crn* genes affect apoptosis in *C. elegans* by conducting time course analyses of embryonic cell corpses (Parrish et al., 2001). We found that RNAi of six genes—*crn-1*, *crn-2*, *crn-3*, *crn-4*, *crn-5*, and *cyp-13*—delayed appearance of embryonic cell corpses during development, generating profiles of embryonic cell corpses similar to that of *cps-6*(RNAi) animals; the peak of embryonic cell corpses was shifted from the bean/comma stage in control(RNAi) animals to the 2-fold stage in these RNAi-treated animals (Figure 2). In contrast, RNAi of four other ORFs (B0438.2, F09G8.2, M02B7.2, and Y57A10A.4) that did not yield any TUNEL phenotype in our screen had no effect on the appearance of embryonic cell corpses in N2 animals (data not shown). Interestingly, *crn-6*(RNAi), like *nuc-1*(RNAi), did not change the profile of cell corpses (Figures 2B and 2G), suggesting that *crn-6* may be dispensable for apoptosis. Since *crn-6* encodes a type II DNase similar to NUC-1, these two nucleases may play a similar role in DNA degradation. We thus tested whether *crn-6*(RNAi) could phenocopy or enhance two other DNA degradation defects associated with the *nuc-1* mutant: failure to digest DNA of ingested bacteria in the intestine and inability of engulfing cells to degrade DNA derived from postembryonic cell deaths, which exists as pycnotic bodies when stained with fluorescent DNA dyes like Syto-11 (Wu et al., 2000). We found that neither *crn-6*(RNAi) nor RNAi of any other *crn* gene or *cyp-13* in either N2 or *nuc-1(e1392)* animals could phenocopy or enhance these two specific DNA degradation defects of *nuc-1* (data not shown), suggesting that these six *crn* genes and *cyp-13* are likely involved in DNA degradation specific for cell deaths.

Since *crn-2*(RNAi) and *crn-3*(RNAi) enhanced the TUNEL phenotype of the *cps-6(sm116)* mutant, *crn-2*/*crn*-3 and *cps-6* appear to function in two independent pathways (Table 1). Intriguingly, more cell corpses were seen at every stage in *cps-6(sm116); crn-2*(RNAi) or *cps-6(sm116); crn-3*(RNAi) embryos than in *cps-6(sm116)*; control(RNAi) embryos (Figure 2I), suggesting that *crn-2*(RNAi) or *crn-3*(RNAi), when combined with *cps-6(sm116)*, may impair cell corpse engulfment (also see below). RNAi of any other *crn* gene or *cyp-13* did not affect the profile of cell corpse appearance in the *cps-6(sm116)* mutant, consis-

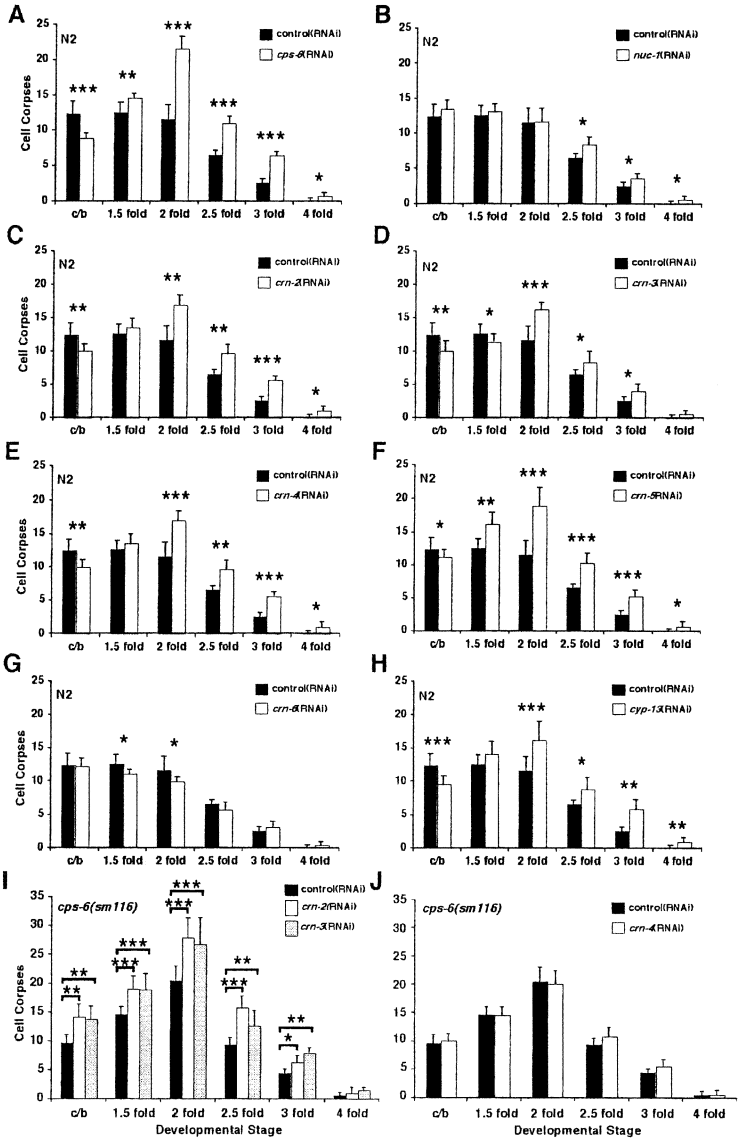

Figure 2. Time-Course Analysis of Embryonic Cell Corpses

L1 larvae from N2 (A–H) or *cps-6(sm116)* (I and J) animals were treated with control(RNAi) or *cps-6*(RNAi) (A), *nuc-1*(RNAi) (B), *crn-2*(RNAi) (C), *crn-3*(RNAi) (D), *crn-4*(RNAi) (E), *crn-5*(RNAi) (F), *crn-6*(RNAi) (G), *cyp-13*(RNAi) (H), *crn-2*(RNAi) or *crn-3*(RNAi) (I), or *crn-4*(RNAi) (J). *crn-1*(RNAi) resulted in a delay in cell corpse appearance that was qualitatively similar to the delay of cell corpse appearance seen in the *cps-6(sm116)* mutant (our unpublished data). Cell corpses were scored at six embryonic stages (comma/bean [c/b], 1.5-fold, 2-fold, 2.5-fold, 3-fold, and 4-fold) from progeny of RNAi-treated animals. The *y* axis represents the mean of cell corpses scored at the head region of embryos (at least 15 animals for each developmental stage), and error bars represent the standard deviation (SD). Control(RNAi) indicates that animals were fed with bacteria containing an expression vector lacking an insert as a negative control. Data derived from control(RNAi) and RNAi treatment of the *crn* genes, *cps-6*, or *nuc-1* at the same stage were compared using an unpaired t test. *$p < 0.05$, **$p < 0.002$, and ***$p < 0.0005$. All other points had p values > 0.05.

tent with the findings that some of these genes (*crn-1, crn-4, crn-5*, and *cyp-13*) function in the same pathway as *cps-6* and some (*crn-6* and *nuc-1*) act at a later stage of DNA degradation (Figure 2J and data not shown).

CRN Nucleases Promote Cell Killing

Because *cps-6* promotes cell killing when assayed in sensitized genetic backgrounds (Parrish et al., 2001), we determined whether the six *crn* genes and *cyp-13* also contribute to cell killing. Like the *cps-6(sm116)* mutation, RNAi of any of the six *crn* genes or *cyp-13* alone has little effect on the deaths of 16 cells that normally occur in the anterior pharynx of animals, generating no or few extra "undead" cell in the assayed region (Table 2; our unpublished data). These results indicate that none of the six *crn* genes or *cyp-13* alone can significantly contribute to cell killing. However, when combined with a weak *ced-3(n2438)* mutation, RNAi of any of these genes except *crn-6* can significantly protect against cell deaths, generating a mean of 2.35–2.88 extra undead cells, compared with a mean of 1.56 extra cells seen in *ced-3(n2438)* animals treated with control(RNAi) (Table 2). A similar enhanced inhibition of cell killing was observed when *crn-2(RNAi)* or *crn-4(RNAi)* was combined with a weak *ced-3(n2447)* mutation (Table 2). These observations indicate that five of the six *crn* genes (except *crn-6*) and *cyp-13* can promote cell killing, just like *cps-6*. We also examined more closely the contributions of *crn-2* and *crn-4* (which function in two different DNA degradation pathways) to cell killing. We found that *crn-2*(RNAi), but not *crn-4*(RNAi), could further increase the number of extra cells observed in *cps-6(sm116); ced-3(n2447)* or *cps-6(sm116); ced-4(n2273)* mutants (Table 2), providing further evidence that *crn-2* functions in a different DNA degradation pathway from *cps-6* and that the two DNA degradation pathways in nematodes can independently promote cell killing.

Recently, it has been shown that each of the two partially redundant cell corpse engulfment pathways in *C. elegans* weakly but independently contributes to cell

Table 2. Multiple *crn* Genes and *cyp-13* Can Contribute to Cell Killing

Strain[a]	No. Scored	Number of Extra Cells[b]		
		Mean ± SEM	Range	p Value[d]
N2; control(RNAi)	16	0	0	n/a
N2; *crn-2*(RNAi)	17	0.06 ± 0.06	0–1	0.17
N2; *crn-3*(RNAi)	19	0.05 ± 0.05	0–1	0.18
N2; *crn-4* (RNAi)	28	0.07 ± 0.05	0–1	0.14
N2; *crn-5*(RNAi)	21	0.05 ± 0.05	0–1	0.18
N2; *crn-6*(RNAi)	20	0	0	n/a
N2; *cyp-13*(RNAi)	15	0.07 ± 0.07	0–1	0.15
ced-3(n2438); control(RNAi)	16	1.56 ± 0.25	0–3	n/a
ced-3(n2447); control(RNAi)	16	1.63 ± 0.25	0–3	n/a
ced-3(n2438); crn-2(RNAi)	17	2.88 ± 0.42	1–6	0.006
ced-3(n2447); crn-2(RNAi)	16	2.63 ± 0.41	0–5	0.02
ced-3(n2438); crn-3(RNAi)	15	2.47 ± 0.32	1–4	0.006
ced-3(n2438); crn-4(RNAi)	16	2.44 ± 0.34	0–5	0.02
ced-3(n2447); crn-4(RNAi)	16	2.82 ± 0.36	1–5	0.005
ced-3(n2438); crn-5(RNAi)	15	2.35 ± 0.26	1–4	0.01
ced-3(n2438); crn-6(RNAi)	15	1.40 ± 0.23	0–3	0.41
ced-3(n2438); cyp-13(RNAi)	15	2.53 ± 0.23	1–4	0.01
cps-6(sm116); ced-3(n2447); control(RNAi)[c]	16	2.63 ± 0.30	1–5	n/a
cps-6(sm116); ced-3(n2447); crn-2(RNAi)[c]	23	3.30 ± 0.28	1–6	0.05
cps-6(sm116); ced-3(n2447); crn-4(RNAi)[c]	15	2.67 ± 0.35	1–6	0.46
cps-6(sm116); ced-4(n2273); control(RNAi)[c]	17	3.65 ± 0.28	2–6	n/a
cps-6(sm116); ced-4(n2273); crn-2(RNAi)[c]	18	4.44 ± 0.31	3–7	0.04
cps-6*(sm116); ced-4(n2273); crn-4*(RNAi)[c]	15	3.80 ± 0.35	1–6	0.36

[a] Control(RNAi) indicates that animals were fed with bacteria containing an expression vector lacking an insert as a negative control.
[b] Extra cells were counted in the anterior pharynx of L3 hermaphrodites using Nomarski optics. Data shown are averages ± SEM.
[c] These strains contain *dpy-5(e61)*.
[d] p values were determined using Student's t tests. Data from RNAi-treated animals were compared to the appropriate RNAi control.

killing (Hoeppner et al., 2001; Reddien et al., 2001). Similarly, we have shown here that each of the two DNA degradation pathways represented by *crn-2* and *cps-6/crn-4* also weakly but independently contributes to cell killing. We thus wanted to determine whether defects in both cell corpse engulfment pathways and both DNA degradation pathways could additively affect cell killing. Interestingly, when the functions of both engulfment pathways and both DNA degradation pathways were reduced by mutations or RNAi, for example, in *cps-6(sm116); ced-7(n1892); ced-5(n1812); crn-2*(RNAi) animals, a mean of 1.2 extra cells was seen, whereas reduction of activity in any of these pathways alone had little effect on cell killing (Table 3). These results indicate that the cell corpse engulfment and the DNA degradation pathways, and possibly other cell death execution pathways, may independently and additively contribute to cell killing.

Defects in Both DNA Degradation Pathways Affect Cell Corpse Engulfment

To verify the intriguing observations that the *cps-6(sm116)* mutation and *crn-2(*RNAi*)* or *crn-3(*RNAi) cause a synthetic defect in cell corpse engulfment, we performed four-dimensional cell lineage analyses to examine the average duration of embryonic cell corpses. In N2 animals treated with control(RNAi), embryonic cell corpses persisted 21.9 min on average (Table 4). *crn-2*(RNAi) treatment of N2 animals or the *cps-6(sm116)* mutation alone did not prolong the persistence of embryonic cell corpses (Table 4). In contrast, *crn-2*(RNAi) treatment of the *cps-6(sm116)* mutant prolonged the

Table 3. DNA Degradation and Cell Corpse Engulfment Pathways Can Additively Contribute to Cell Killing

Strain[a]	No. Scored	Number of Extra Cells[b]	
		Mean ± SEM	Range
cps-6(sm116); control(RNAi)	18	0.06 ± 0.06	0–1
ced-5(n1812); control(RNAi)	20	0.07 ± 0.06	0–1
ced-7(n1892); control(RNAi)	25	0.12 ± 0.08	0–1
N2; *crn-2*(RNAi)	17	0.06 ± 0.06	0–1
cps-6(sm116); ced-7(n1892); ced-5(n1812); crn-2(RNAi)[c]	20	1.20 ± 0.15	0–2

[a] RNAi experiments were carried out as described in Experimental Procedures. Control(RNAi) indicates that animals were fed with bacteria containing an expression vector lacking an insert as a negative control. *cps-6* and *crn-2* represent two different DNA degradation pathways. *ced-5* and *ced-7* represent two different cell corpse engulfment pathways.
[b] Extra cells were counted in the anterior pharynx of L3 hermaphrodites using Nomarski optics. Data shown are averages ± SEM.
[c] This strain contains *dpy-5(e61)*.

Table 4. Inactivation of Both *cps-6* and *crn-2* Prolongs the Persistence of Cell Corpses

Strain[a]	Corpse Duration[b] (n)	Duration of Cell Divisions[b,c]
N2; control(RNAi)	21.9 ± 1.2 (7)	85.0
N2; *crn-2*(RNAi)	24.5 ± 0.8 (5)	91.5
cps-6(sm116); control(RNAi)	23.4 ± 1.3 (7)	93.0
cps-6(sm116); crn-2(RNAi)	34.0 ± 1.9 (7)	91.5

[a] RNAi experiments were carried out as described in Experimental Procedures.
[b] Corpse duration and duration of cell divisions are in minutes. At least three animals from each strain were examined, and results from one representative animal are shown. "n" indicates the number of cell corpses examined in one embryo. Data represented are averages ± SEM.
[c] The duration of four cell divisions in the MS cell lineage from the MS cell to the MSpppp cell (Sulston et al., 1983) was followed in embryos monitored for the duration of cell corpses.

persistence of embryonic cell corpses by 55%, indicative of a defect in cell corpse engulfment (Table 4). To rule out the possibility that the observed differences in corpse durations resulted from different rates of development in different animals, we simultaneously measured the durations of four cell divisions in the MS cell lineage (Sulston et al., 1983) and found them to be similar in all embryos analyzed (Table 4). This unexpected, synthetic engulfment defect displayed by the *cps-6(sm116)*; *crn-2*(RNAi) animals may reveal a significant, intrinsic connection between the apoptotic DNA degradation process and the cell corpse recognition/engulfment process.

Multiple CRN Nucleases and CPS-6 Likely Interact to Form a DNA Degradation Complex

To better understand how these nine nucleases affect apoptotic DNA degradation in nematodes, we examined potential protein-protein interactions among these nucleases using Glutathione-S transferase (GST) fusion protein pull-down assays. Interestingly, we have identified a number of in vitro interactions among proteins functioning in the *cps-6* pathway (Figure 3; see Supplemental Figure S3 at http://www.molecule.org/cgi/content/full/11/4/987/DC1), suggesting that five nucleases in this pathway (CPS-6, CRN-1, CRN-4, CRN-5, and CYP-13) may function together, possibly in a large complex along with nonnuclease components such as WAH-1 (Wang et al., 2002), to promote apoptotic DNA degradation in vivo; we have named this multinuclease complex the "degradeosome." Only one interaction, CRN-3/CRN-5, was identified between proteins functioning in two different DNA degradation pathways, and since their corresponding mammalian homologs interact in the exosome (Brouwer et al., 2001), this interaction may be important for their shared role in RNA processing. No strong interactions were observed between NUC-1 or CRN-6 and proteins acting in either the *cps-6* or *crn-2* pathways, consistent with NUC-1 and CRN-6 functioning in later stages of the DNA degradation process or in their own DNA degradation pathways.

Discussion

Multiple Components and Pathways Participate in the Execution of Apoptotic DNA Degradation in *C. elegans*

Using the TUNEL assay and an RNAi-based functional genomic screen, we have identified seven additional genes involved in *C. elegans* apoptotic DNA degradation. RNAi of each of these new genes leads to an accumulation of TUNEL-reactive DNA intermediates in apoptotic cells and, in most cases (except *crn-6*), a delay of cell corpse appearance during embryonic development and inhibition of cell killing in sensitized genetic backgrounds, confirming their importance for apoptosis. The identification of seven apoptotic nucleases in addition to two previously known ones, NUC-1 and CPS-6, indicates that the apoptotic DNA degradation process is more complicated than we originally expected. Indeed, we found that two of these genes, *crn-2* and *crn-3*, function in a DNA degradation pathway distinct from the one that includes *cps-6* and four other genes (*crn-1, crn-4, crn-5*, and *cyp-13*) and that these two pathways can independently contribute to apoptotic DNA degradation and cell killing in *C. elegans*. As for *crn-6* and *nuc-1,* both encode homologs of DNase II and appear to be dispensable for apoptosis. They likely function in different DNA degradation pathways or at later stages of the DNA degradation process (our unpublished data).

Based on the results of the TUNEL assays, cell corpse assays, and extra cell counts, *cps-6, crn-1, crn-4, crn-5,* and *cyp-13* appear to function in the same DNA degradation and cell death pathway, along with *wah-1*, the *C. elegans* AIF homolog that we identified recently (Wang et al., 2002). Among them, CPS-6 and CYP-13 are endonucleases, CRN-1 is a structure-specific endonuclease and a 5′-3′ exonuclease (J.Z.P., B.H. Shen, and D.X., unpublished data), and CRN-4 and CRN-5 are homologous to 3′-5′ exonucleases. The finding that these five nucleases can interact with one another in vitro raises the interesting possibility that they may form a multinuclease complex in vivo to execute chromosome fragmentation/degradation, utilizing their endo- and exonuclease activities in a cooperative manner. Such a DNA degradation complex, which we named the "degradeosome," could be analogous to the exosome, a multiexonuclease complex that specializes in RNA processing and degradation (Brouwer et al., 2001). Since WAH-1 also binds to CPS-6 and enhances its nuclease activity, WAH-1 is likely a nonnuclease component of the degradeosome (Wang et al., 2002). Furthermore, we found that several *cps* genes (CED-3 *p*rotease *s*uppressors) that do not map to any of the *crn* gene loci also function in the *cps-6* pathway (our unpublished data). These *cps* genes could encode additional components of the degradeosome.

It is intriguing that RNAi of six *crn* genes, *cps-6*, or *cyp-13* did not produce two typical DNA degradation

A

GST-fusion proteins

^{35}S-proteins	GST	CPS-6	NUC-1	CRN-1	CRN-3	CRN-4	CRN-5	CRN-6	CYP-13
Luciferase	-	-	-	-	-	-	-	-	-
CPS-6	-	+	-	++	-	++	+	-	+
NUC-1	-	-	-	-	-	-	-	-	-
CRN-1	+	++	+	+	+	++	+	+	++
CRN-2	-	-	-	-	-	-	-	-	-
CRN-3	+	+	+	+	++	+	++	+	+
CRN-4	-	+	+	+	-	++	++	-	++
CRN-5	-	+	+	-	-	++	++	-	++
CRN-6	-	-	-	-	-	-	-	-	-
CYP-13	-	++	-	+	-	++	++	-	++

B

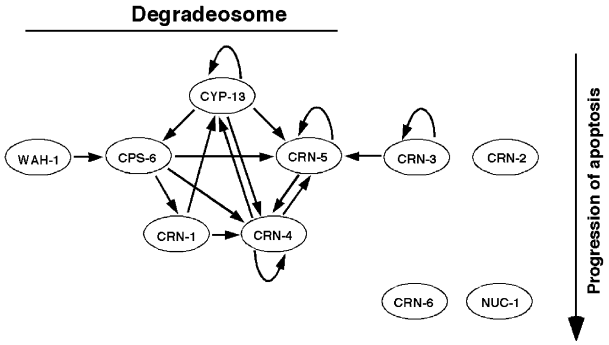

Figure 3. Analysis of In Vitro Interactions among *C. elegans* Apoptotic Nucleases

(A) Summary of in vitro interactions among CPS-6, NUC-1, CPY-13, and six CRN proteins. Interactions between proteins were examined using GST fusion protein pull-down assays and were evaluated relative to background binding of ^{35}S-Methionine-labeled Luciferase to GST-fusion proteins and ^{35}S-Methionine-labeled proteins to GST (see Supplemental Figure S3 at http://www.molecule.org/cgi/content/full/11/4/987/DC1). "−" indicates no detectable binding, "+" indicates an interaction consistently observed above background levels, and "++" indicates a strong interaction. GST-CRN-2 is not shown because we have not been able to purify it. The CRN-1/CPS-6 interaction has been characterized in detail (J.Z.P., B.H. Shen, and D.X., unpublished data).

(B) Interaction map for nucleases involved in apoptotic DNA degradation in *C. elegans*. An arrow indicates an interaction between a GST-fusion protein (pointed by the arrow) and a ^{35}S-Methionine-labeled protein. For example, GST-CPS-6 bound ^{35}S-CYP-13. Only strong protein interactions are depicted. The interaction between CPS-6 and WAH-1 was described previously (Wang et al., 2002). NUC-1 and CRN-6 likely function at later stages of apoptosis.

phenotypes seen in the *nuc-1* mutant, retention of ingested bacterial DNA in intestines and the presence of condensed, Syto 11-reactive material (pycnotic bodies) derived from undegraded dying cell DNA in engulfing cells (Wu et al., 2000). These observations are consistent with our findings that *nuc-1* functions in a different DNA degradation process from the other eight apoptotic nucleases and is not a cell death-specific nuclease (Table 1; Parrish et al., 2001). We found that *cps-6*-mediated DNA degradation occurs early during apoptosis and appears to precede and be important for chromatin condensation (our unpublished data). Thus, TUNEL-reactive DNA degradation intermediates in *cps-6*-defective animals may not be condensed enough to be stained strongly by Syto 11. In contrast, *nuc-1* appears to act in later stages of DNA degradation after chromatin condensation occurs (our unpublished data), and the pycnotic bodies are likely condensed TUNEL-positive DNA degradation intermediates that normally are resolved by NUC-1. In support of this possibility, we found that accumulation of pycnotic bodies in *nuc-1(e1392)* mutants is completely dependant on *ced-1* activity just like the TUNEL phenotype of *nuc-1* (our unpublished data). Further investigation of the functional sites and stages of these nine apoptotic nucleases will be important to understand how they act to mediate apoptotic DNA degradation.

Apoptotic DNA Degradation Affects Cell Corpse Engulfment

DNA degradation and cell corpse engulfment are two distinct processes during cell death execution and appear to proceed simultaneously during apoptosis. Some recent studies have indicated that the phagocytosis process may contribute significantly to the DNA fragmentation process in apoptotic cells (McIlroy et al., 2000; Platt et al., 1998). For example, a mammalian lysosomal acid nuclease in macrophages has been suggested to directly mediate degradation of chromosomal DNA of apoptotic cells after they are engulfed by macrophages (McIlroy et al., 2000). In addition, mutations in the *ced-1* or the *ced-7* gene, both of which are important for cell corpse engulfment in *C. elegans*, abolish or significantly reduce the TUNEL phenotype of the *nuc-1(e1392)* mutant (Wu et al., 2000), suggesting that *ced-1* and *ced-7* may regulate the generation of TUNEL-positive DNA breaks resolved by NUC-1. Thus there is likely an intrinsic connection between the DNA degradation process and the cell corpse engulfment process. In our studies, we found that defects in both the *crn-2/crn-3* and *cps-6* DNA degradation pathways resulted in persistence of cell corpses, suggesting that the DNA degradation process may actually affect the cell corpse engulfment process. At present, we do not know the mechanistic basis for such an observation. It is possible that chromosomal DNA, after being fragmented and degraded, is released or presented as an "eat-me" signal for phagocytosis. In support of this hypothesis, it has been reported that nucleosomes generated by cleavage of chromosomal DNA in apoptotic cells are released from the cells to activate immune responses (Bell et al., 1990); such nucleosomes could be recognized by phagocytic cells. Alternatively, given that both *cps-6* and *crn-2/3* pathways are important for normal progression of apoptosis and can contribute to cell killing, the DNA degradation process may need to proceed to a certain stage before some of the eat-me signals can be made and presented. In either case, our finding further suggests that the DNA degradation process and the cell corpse engulfment

process may regulate one another to efficiently promote the killing, degradation, and removal of apoptotic cells.

Apoptotic DNA Degradation and Autoimmune Disorders

In addition to its roles in promoting cell killing and cell corpse engulfment, apoptotic DNA degradation has been proposed to play an important role in higher organisms to remove highly antigenic DNA or nucleosomes from apoptotic cells and prevent them from eliciting autoimmune responses (Stollar, 1989; Zhang and Xu, 2002). In fact, a number of human autoimmune disorders, including lupus, are characterized by high concentrations of circulating DNA that may result from failure to properly execute apoptotic DNA degradation (Fournie, 1988; Suzuki et al., 1997). In addition, mice deficient in DNase I, a nuclease that serves to digest extracellular DNA or chromatin released at sites of high cell turnover or apoptosis, develop classical symptoms of systemic lupus erythematosus (Napirei et al., 2000; Walport, 2000), further supporting the possible connection between apoptotic DNA degradation and autoimmune disorders. Interestingly, autoantibodies against PM-Scl100 and Rrp46, mammalian homologs of CRN-3 and CRN-5, respectively, are often found in patients with scleroderma, polymyosistis/scleroderma overlap syndrome, and idiopathic inflammatory myopathy (Brouwer et al., 2002). However, the etiopathogenesis of these syndromes is poorly understood. Given our finding that CRN-3 and CRN-5 are important for apoptotic DNA degradation, we suggest that inactivation of PM-Scl100 or Rrp46 by autoantibodies or genetic mutations could compromise apoptotic DNA degradation, providing a source of undegraded DNA that either elicits or augments autoimmune responses. Similarly, inactivation by genetic mutations (or other means) of the other apoptotic nucleases, including human homologs of the other *crn* genes, *cyp-13*, and *cps-6,* may also contribute to the progression of autoimmune disorders. Gene targeting of corresponding mouse CRN homologs will help answer these questions.

Multiple Nucleases Likely Play Dual Roles in Cell Survival and Cell Death

One emerging theme in cell death regulation is that some components important for cell growth and survival are "transformed" into proapoptotic molecules during apoptosis. The best example is cytochrome c, which normally plays an important role in oxidative phosphorylation in mitochondria and participates in production of ATP (Liu et al., 1996; Reed, 1997). However when it is released from mitochondria, it binds to and activates Apaf-1 to catalyze the activation of pro-caspase-9 (Zou et al., 1997, 1999). Recently, AIF, a mitochondrial oxidoreductase, has been implicated in preventing oxidative stress in normal cells but becomes a proapopotic protein when it is released from mitochondria during apoptosis (Klein et al., 2002; Susin et al., 1999). In our studies, we found that several CRN proteins are homologous to components of important cellular processes such as RNA processing (*crn-3*, *crn-4*, and *crn-5*), protein folding (*cyp-13*), and DNA replication and repair (*crn-1*) and appear to be important for the survival and proper development of the nematode (*crn-1, crn-3,* and *crn-5*). In the case of CRN-1, which we have studied in the most detail, we have confirmed that CRN-1 plays a dual role in both cell survival and cell death in *C. elegans* (J.Z.P., B.H. Shen, and D.X., unpublished data). Thus, these CRN proteins likely behave as double agents, like cytochrome c, to mediate normal cellular functions in living cells and proapoptotic functions in dying cells.

Functional Genomic Screen Is an Effective, Alternative Approach to Identify New Cell Death Components

Using this systematic, genome-wide screen, we believe that we have identified most of the apoptotic nucleases in *C. elegans*, which would not be possible using conventional genetic screens, given the subtle cell death phenotypes displayed by these RNAi-treated animals and other mutants defective in DNA degradation (Parrish et al., 2001; Wang et al., 2002; Wu et al., 2000). Even sensitized genetic screens, such as the one used to identify *cps-6* (Parrish et al., 2001), will miss genes like *crn-1*, whose loss-of-function phenotype is embryonic lethal, or identify genes whose genetic characterization turns out to be laborious (J.Z.P., X.C. Wang, and D.X., unpublished data). Given the nature of this candidate-based functional genomic approach, the effectiveness of the screen is limited by the incomplete annotation of the *C. elegans* genome and the possibility that a nuclease may lack a canonical nuclease motif, as in the case of DFF40/CAD (Enari et al., 1998; Halenbeck et al., 1998; Liu et al., 1998, 1997). Furthermore, our screen may miss genes that function redundantly to mediate DNA degradation and genes that are insensitive to RNAi. These may account for why we have not identified the nuclease(s) responsible for creating TUNEL-positive DNA breaks during apoptosis. Nevertheless, the identification of seven new genes involved in multiple apoptotic DNA degradation pathways indicates that the process is far more complex than expected, and our studies will provide an important starting point for systematic analysis of the underlying mechanisms of apoptotic DNA degradation as well as the mechanisms by which important "housekeeping" components are recruited to participate in the execution of cell death.

Experimental Procedures

C. elegans Strains and Culture Conditions

C. elegans strains were maintained using standard procedures (Brenner, 1974). All strains used in this study have been described previously (Parrish et al., 2001; Riddle et al., 1997).

RNAi Screen

For each ORF, we cloned a partial cDNA (>200 bp) into a bacterial dsRNA (double-stranded RNA) expression vector (pPD129.36, kindly provided by A. Fire), introduced the expression vector into a bacterial host, TH115, and carried out RNAi experiments using a bacterial feeding protocol (Parrish et al., 2001). Briefly, L1 larval animals were fed with bacteria expressing a specific dsRNA. The progeny of the treated animals were then scored for TUNEL and other cell death phenotypes. RNAi results for approximately 20% of the ORFs were verified using dsRNAs corresponding to the full-length cDNAs, and no differences in phenotypes were noted. Effects of RNAi of each ORF were tested in three genetic backgrounds, N2 (wild-type), *cps-6(sm116)*, and *nuc-1(e1392)*, to identify genes that generate or resolve TUNEL-positive ends and to eliminate false

positives. To further rule out false positives, ORFs that gave rise to TUNEL-phenotypes following RNAi treatment were retested in triplicate.

Quantification of Cell Corpses and Extra Cells

The number of cell corpses in the head region of living *C. elegans* embryos and the number of extra cells in the anterior pharynx of L4-stage hermaphrodites were counted using Nomarski optics as previously described (Parrish et al., 2001).

TUNEL Assays

TUNEL assays were carried out as described previously (Parrish et al., 2001) using an in situ cell death detection kit (Roche).

4D Microscopy

Early *C. elegans* embryos (one to four cell stage) were mounted on slides with agar pads in M9, and coverslips were sealed with mineral oil. Images in a 15 micron z series (1 micron/each layer) were captured every 30 s for 500 min using a Leica Nomarski microscope equipped with a Cohu CCD camera and Scion image 1.62c software. Images were compiled into a viewable 4D movie using a 4D Turnaround software and viewed using 4D Viewer.

Molecular Biology

Standard procedures were used for polymerase chain reaction (PCR) amplification, sequencing, and subcloning. Oligonucleotide sequences used in this study are available upon request. cDNAs corresponding to the tested ORFs were cloned into the pPD129.36 vector via its NheI and XhoI sites or the XhoI site alone (details available upon request). Full-length cDNAs were subcloned into the pGEX4T-2 vector for generating GST fusion proteins and into the pcDNA3.1 vector for in vitro transcription/translation experiments.

To obtain full-length cDNA clones corresponding to various ORFs, we isolated total RNA from mixed-stage wild-type animals using TRI-Reagent (Sigma) at a ratio of 10:1 (TRI-Reagent:pelleted worms) and then PCR amplified the cDNAs using an Enhanced Avian RT-PCR kit (Sigma). Briefly, Oligo-dT primers were used to reverse transcribe 10 μg of total RNA, and sequence-specific primers were used to amplify cDNAs by PCR from the resulting pool of first strand cDNAs. Two *crn-6* cDNAs (700 bp and 1.1 kb) were isolated using RT-PCR. The shorter cDNA clone is identical to *yk720e2*, a cDNA clone provided by Y. Kohara. The longer cDNA clone corresponds to the predicted *crn-6* ORF and was used to make the GST fusion protein for protein binding studies.

Protein Expression and Purification

^{35}S-Methionine-labeled proteins were synthesized using the Promega TNT rabbit reticulocyte lysate system as instructed by the manufacturer. GST-fusion proteins were prepared by growing bacterial BL21(DE3)pLysS cells harboring the expression vector to an OD_{595} of ~0.6 and then inducing the expression of the fusion protein with 0.2 mM IPTG for 12 hr at 15°C. Cells were harvested by centrifugation and lysed in the PBS buffer via sonication. Following centrifugation of the lysate, supernatant was incubated with Glutathione Sepharose resin (Amersham). Bound proteins were washed extensively using PBST buffer (PBS buffer with 1% Triton X-100) and eluted in PBST containing 20 mM reduced glutathione. Eluted proteins were dialyzed against buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, and 15% glycerol and were stored at −80°C.

GST Pull-Down Assays

10 μg of the purified GST fusion protein was incubated with 5 μl ^{35}S-Methionine-labeled proteins in PBST (0.5% Triton X-100) at 4°C for 1 hr. 10 μl of Glutatione Sepharose resin were then added to each reaction and allowed to equilibrate with the proteins at 4°C for one additional hour. The resin was washed three times (10 min each) with PBST (1% Triton X-100), and the bound proteins were eluted with sample buffer and resolved on a 10% SDS-polyacrylamide gel, which was then fixed and dried before being subjected to Phosphorimager analysis.

Nuclease Assays

An appropriate amount of purified protein was incubated with 1 μg of plasmid DNA in 20 mM HEPES (pH7.5), 10 mM NaCl, 3 mM $MgCl_2$, 1 mM DTT, 1 mM $CaCl_2$ and 2% glycerol at 37°C for 2 hr. The reactions were then resolved on a 1.5% agarose gel and visualized with ethidium bromide.

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